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14. ABSTRACT
The project proposes a novel preclinical approach to study risk factors for and neurobiology of post-traumatic stress and depression. Why risk factors? Because PTSD is the only psychiatric disorder to which there is seemingly a clear etiological agent- a traumatic event that triggers it, most animal models of PTSD concentrate on what would constitute a trauma in the studied animals. However, because the majority of people exposed to traumatic experiences actually develop PTSD the exposure to the traumatic experience is necessary, but no a sufficient condition to induce disorder. WE focus on both distal (childhood adversities) and proximal (sleep restriction) potential risk factors, with high relevance to soldiers.

1. To establish an effective animal model of PTSD that would consider the contribution of risk factors to the introduction of PTSD
2. To examine the role of sleep restriction as a proximal risk factor of PTSD
3. To establish the role of childhood adversities as a long-term risk factor in PTSD, particularly in association with sleep restriction.
4. To develop the model as a platform for pharmacological testing of novel targets for drug development

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Title: **Early life stress and sleep restriction as risk factors in PTSD – An integrative preclinical approach**

Table of contents:

Introduction

The project proposes a novel integrative preclinical approach to study risk factors for and neurobiology of post-traumatic stress and depression.
Why risk factors? - Because PTSD is the only psychiatric disorder to which there is seemingly a clear etiological agent – a traumatic event that triggers it, most models of PTSD concentrate on what would constitute a trauma in the studied animals. However, because the majority of people exposed to traumatic experiences actually do not develop PTSD the exposure to the traumatic experience is necessary, but not a sufficient condition to induce the disorder. We focus on both distal (Childhood adversities) and proximal (Sleep restriction) potential risk factors, with high relevance to soldiers. The primary aims of the project are thus:

1) To establish an effective animal model of PTSD that would consider the contribution of risk factors to the induction of the trauma.
2) To examine the role of sleep restriction as an immediate risk factor in PTSD.
3) To establish the role of childhood adversity as a long-term risk factor in PTSD, particularly in association with sleep restriction.
4) To develop the model as a platform for pharmacological testing of novel targets for drug development.

Body

Task 2. **The establishment of sleep restriction methodology in Haifa –**

+

Task 4: **The impact of sleep restriction on the outcome of exposure to UWT-**

As was reported previously, we have adopted the well established protocol of Meerlo et al, (2008). In order to validate that this model works well in our hands, we have conducted a telemetry study, using a combination of a DSI telemetry system and the sleep restriction wheels system we have set for this project. Results of that study indicated that this sleep restriction protocol had short-term effects of body temperature and activity. This was taken as sufficient in order to move forward and to examine whether sleep restriction prior to the exposure to the UWT would worsen the long-term effects of the trauma (Task 4).

Initial results indicated a limited effect for sleep restriction in the way it was inflicted here, on the long-term behavioral outcome of the UWT. The results could indicate that contrary to our hypothesis, sleep restriction is not a proximal risk factor for developing PTSD. However, because of the potential significance of such a conclusion, we, following a discussion with our colleagues at the Walter Reed Army Institute of Research, decided to examine a modification of the original Peerlo et al, 2008 protocol, before a final conclusion is made.

Rather than having 4 hours of normal home cage rest each day, rats were given only 2 hours to rest in their home cages each day, thus staying 22 hours on the wheels. In addition we also examined the possibility that SR would have a long term effect on the reaction to UWT when the sleep is restricted following the UWT and not before.

Aim: Thus the aims of this experiment were:

1. To examine whether SR exacerbates the long term reaction to UWT, when it is applied either prior to the UWT or following it.
2. To test the effects of a more severe SR protocol.
3. To control for possible masking stressors by minimizing animals' isolation and not recording body temperature and activity level to avoid the stress of surgery.

Methods

Animals

Male Sprague Dawley rats (~8 weeks old, 250-275g) were used for the experiment. Animals were group housed at 22 ± 2°C under 12-h light/dark cycles. Water and food were available ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

Experimental groups

All rats were randomly assigned to one of the following experimental conditions:

1. SR prior to UWT (SRU) – Rats exposed to SR, followed by UWT.
2. SR Control prior to UWT (CU) – Rats exposed to the control procedure of SR, followed by UWT.
3. SR following UWT (USR) – Rats exposed to UWT, followed by SR**.**
4. SR control following UWT (UC) - Rats exposed to UWT, followed by the control procedure of SR**.**
5. UWT alone (U) – Rats exposed to UWT alone, with no procedure of SR.

- This group was used because of technical problem with some of the activity wheels during the running of experiment; hence it has small number of animals and was not used in the statistical analysis.

Experimental design

Following delivery and acclimation to the vivarium, all rats excluding group U were habituated to the SR apparatus by placing them on the wheels for 1 h on 3 consecutive days (slowly or voluntary rotating wheels, according to the experimental group). Following wheels habituation, rats from groups USR, UC and U were exposed to UWT. Then some of the rats were exposed to SR for 8 days and the others were exposed to the control procedure of SR (excluding rats from group U,

which stayed in their home cages). After 8 days of SR, rats from groups SRU and CU were exposed to UWT. 4 weeks following UWT, rats were assessed for anxiety level using open field, elevated plus maze and acoustic startle. Timeline of procedures is presented in figure 10.

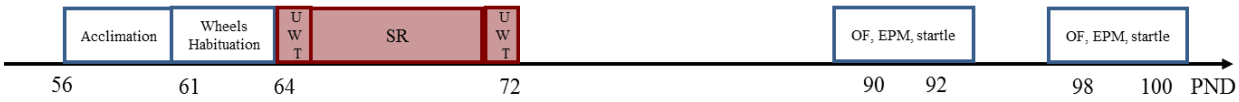

Figure 10: Timeline of procedures.

Procedures and assessments

Sleep restriction

The same procedure as described in the first experiment, only SR was more severe. Rats were allowed to sleep in their home cages only between 8:00-10:00, the first 2 hrs of the light phase. The remaining 22 hrs of the day, they were on the wheels.

Acoustic startle testing

Unconditioned startle response to an acoustic stimulus was measured using a standard startle chamber (Panlab SLU, Spain). Rats were held using a plastic cylinder in the startle apparatus. The apparatus is equipped with a speaker for producing sound bursts and with a high sensitivity weight transducer system that allows recording and analysis of the signal generated by the rat movement during each sound burst. Output from the transducer is led to a computer for sampling.

The protocol was adapted from Adamec et al. (2012). Prior to startle testing, animals were acclimatized to the darkened apparatus for 5 min with a background white noise level of 60 dB. Following acclimatization, rats received a 50 ms burst of 120 dB rising out of the 60 dB background noise once every 30 s for 15 min. The 30 trials were conducted in the dark.

Statistical Analysis

Differences were determined using Repeated measures analysis of variance (ANOVA) and T-test for independent samples.

Results

Activity levels while on the activity wheels during the SR protocol

As depicted in figure 11, Repeated measures ANOVA indicated a significant difference in the average rotations per hour on the activity wheels during 8 days of SR only during the light phase for both rats that were exposed to SR protocol prior to UWT [$F_{(1,25)}$= 10.69, $p<0.01$] or following it [$F_{(1,25)}$= 12.79, $p<0.01$]. Apparently during the light phase SR groups were forced to be more active than the control groups, but during the dark phase there was no difference in the general level of activity.

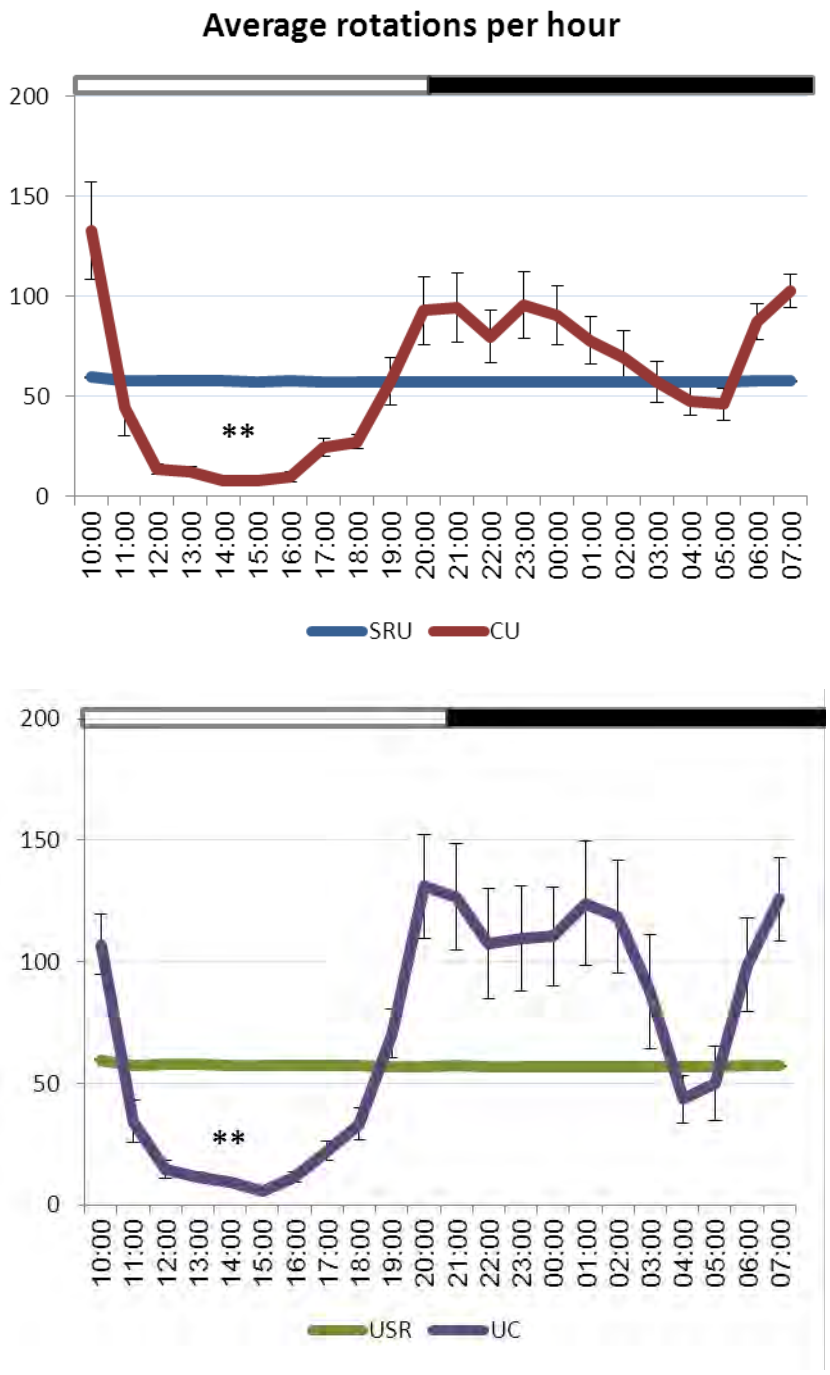

Figure 11: Rotations per hour in the activity wheels during 8 days of SR**:** During the light phase the SR groups were forced to be more active than the control groups but there was no difference between groups during the dark phase. The horizontal white and black bars represent light and dark, respectively.

[N: SRU - 11, CU – 16, USR - 11, UC - 16. (** significant difference between groups, p<.01)].

The long term behavioral effects of SR on the reaction to UWT

Open field test

T-test indicated a significant difference on the number of entries to the central area of the open field, between the groups that were exposed to the SR protocol prior to UWT [$t_{(25)}$=-2.06, p≤0.05].

As indicated in figure 12, 4 weeks after the exposure to UWT, rats that were sleep restricted prior to UWT, entered the central area of the open field fewer times than controls. There were no other significant differences between groups that were exposed to SR protocol prior to UWT or following it.

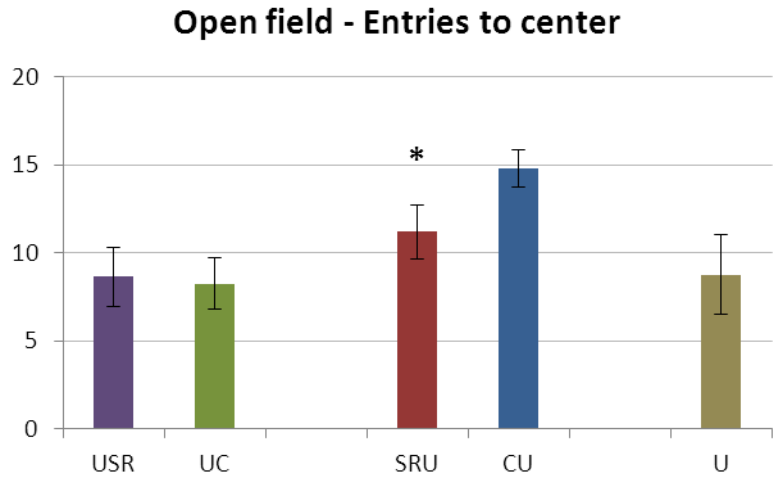

Figure 12: Number of entries to the central area of the open field test: Rats that were sleep restricted prior to UWT, entered the central area of the open field less times than controls, 4 weeks after the exposure to UWT. There were no other significant differences between groups.
[N: USR – 11, UC – 16, SRU - 11, CU – 16, U - 4. (* significant difference compared to CU, $p \leq .05$)].

Elevated plus maze test

No significant differences were found between groups, in none of the measures.

Acoustic startle response test

T-test indicated a significant difference between the rats that were sleep restricted before the UWT (SRU) and their controls (CU) in the average of their maximal amplitude of startle [$t_{(17)}$=-2.15, $p<0.05$]. Additionally, Repeated measures ANOVA indicated a significant difference between the rats that were sleep restricted before the UWT (SRU) and their controls (CU) in their maximal amplitude of startle response to the repeated sound bursts [$F_{(1,17)}$= 4.63, $p<0.05$]. 4 weeks following UWT, sleep restricted rats had smaller maximal amplitude of startle response, in average as depicted in figure 13, as well as repeatedly, as depicted in figure 14. Apparently sleep restricted rats actually showed a stronger habituation to the sound bursts. No significant differences were found between the groups that went through the SR protocol following UWT.

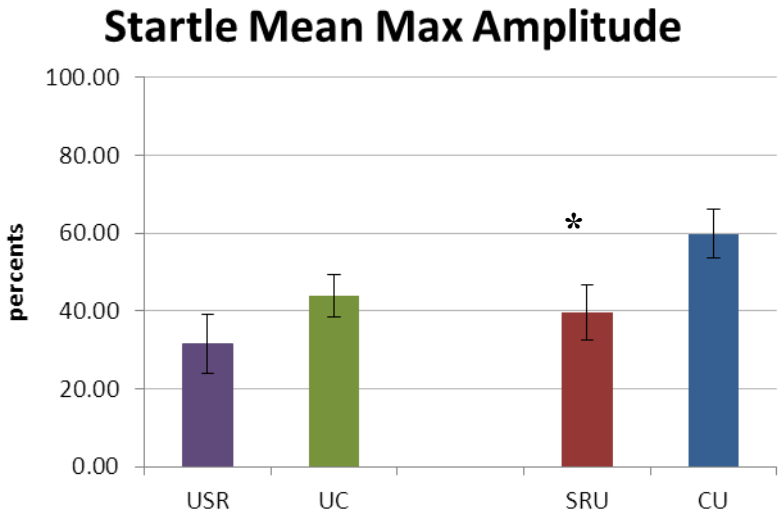

Figure 13: Average of maximal amplitude of startle response: Rats that were sleep restricted prior to UWT show lower maximal amplitude in average, compared to their controls, 4 weeks after the UWT. No differences were found between rats in the groups that went through the SR protocol following the UWT.

[N:, SRU - 8, CU – 11, USR – 7, UC – 11. (* significant difference compared to CU, $p<.05$)].

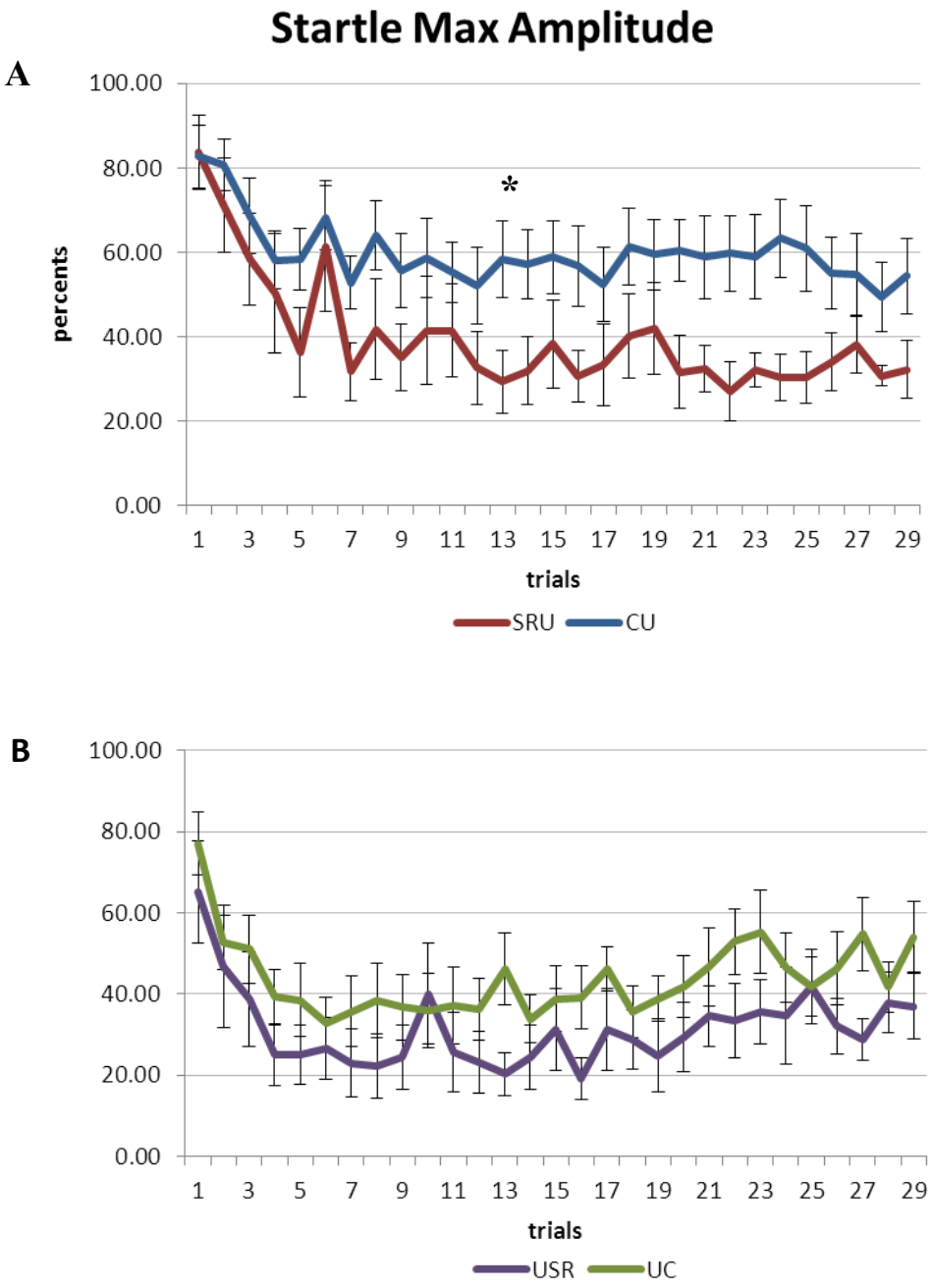

Figure 14: Maximal amplitude of startle response: Rats that were sleep restricted prior to UWT show lower maximal amplitude through the different trials, compared to their controls, 4 weeks after the UWT [**A**]. No differences were found between rats in the groups that went through the SR protocol following the UWT [**B**].
[N:, SRU - 8, CU – 11, USR – 7, UC – 11. (* significant difference between groups, $p<.05$)].

Summary of Results

SR following UWT had no significant effect in the parameters that were measured.

However, using a more severe form of the SR protocol, showed some behavioral effects for SR prior to UWT on the long term reaction to UWT. In the open field, rats that were sleep restricted

prior to UWT entered the central area fewer times than controls. Additionally, in the acoustic startle response test, rats that were sleep restricted prior to UWT, showed a stronger habituation to the sound bursts.

These results were obtained despite the fact that other potential stress factors (implantation of the telemetry transmitters and minimizing isolation) were eliminated.

We have initiated a discussion with our colleagues as well as with Dr. Thomas Nylen from UCSF (an expert on sleep and PTSD), about the implications of these findings and how they should guide the continuation of this aspect of the project. Several ideas are considered, including to test whether the relatively mild effect of SR here is due to the relatively low level of associated stress, as it is known that sleep deprivation has much more sever effect when associated also with stress. Another aspect to be considered is the possibility of returning to 4 hours of rest in home cage but non-continuous 4 hours, in order to simulate closer the conditions of deployed units. Finally, the question of which behavioral tests may be added will be discussed.

In any case, the result of these discussions is that aspects of TASK 2+4 will continue to be studied also in years 3-4.

Task 3: **Behavioral and neurobiological investigation of the impact of the under-water trauma (UWT) with or without a reminder cue –**

As was reported within the 1st annual report for task 5, we found that the impact of whether or not the individual was exposed to 'juvenile' stress was far more significant to the outcome compared with whether or not they were introduced to a trauma reminder. We now have started to utilize a more detailed analysis of individual behavior of exposed animals and preliminary findings indicate that with this analysis it is possible to demonstrate an added contribution of the reminder cue, even though the 'juvenile' stress pre-exposure is still the most significantly affecting factor.

Because of that we are progressing in two directions –

a) We continue to develop the novel individual analysis approach and to examine results based in this type of analysis. This is work-in-progress and we will continue to examine the issue of the impact of a reminder cue with this approach also in years 3-4. A preliminary version of this approach was published, with the indication of the support of the DOD (Horovitz et al, 2012), and so will not be detailed here.
b) We mainly focus on examining the outcome of exposure to a combination of juvenile stress + adulthood trauma, 4 weeks after the exposure to the trauma in adulthood, as will be further detailed in the report on task 5, below.

c) We have run an expression study on animals that have been exposed only to the UWT, to start identifying potential target pathways for planning drug intervention. This study has been published, with the indication of the support of the DOD (Sood et al, 2012), and so will not be detailed here.

Task 5: **The impact of pre-exposure to juvenile stress on the outcome of exposure to UWT –**

Introduction

The UWT has been developed as a unique model of acute robust trauma and has been demonstrated to have long-lasting behavioral consequences with strong face validity to PTSD symptoms (Richter-Levin, 1998; Cohen et al. 2009). More recently an additional dimension has been added to this model – the impact of exposure to a reminder cue, which was found to have clear consequences at the behavioral and electrophysiological levels (task 3, above). We continue to investigate the behavioral, electrophysiological and biochemical associated alterations, also in order to establish the baseline for assessment of the effects of predisposing factors later on.
At the end of the first year we have finished running a large scale experiment, have analyzed the behavior and concluded that –

- First, by itself, UWT had effects even 4 weeks after the exposure to the trauma, as was indicated by moderate symptoms exhibited by the animals in the Elevated Plus Maze. The longevity if the effects of the trauma are very important for this to be accepted as a PTSD-relevant model.
- Introducing an odor reminder cue, which was not part of the trauma context, during the behavioral tests 4 weeks after the exposure to the trauma, had no added impact by itself.
- Prior exposure of the animals to a distal risk factor (the juvenile stress) had a moderate added impact, as was indicated by more severe symptoms in the Elevated Plus Maze test and a similar tendency in the Open Field test.
- However, prior exposure of the animals to a distal risk factor (the juvenile stress) had a moderate added impact on the effect of the reminder cue. Animals that were exposed to the combination of the distal risk factor and UWT, exhibited, 4 weeks after the exposure to the UWT trauma, significantly more severe symptoms in the presence of the reminder cue, as was indicated by more severe symptoms in both the Elevated Plus Maze test and the Open Field test.

Thus, even before adding the question of the impact of the combination of distal (juvenile stress) and proximal (sleep restriction) risk factors on coping with the trauma, this protocol - of prior exposure to Juvenile test, exposure in adulthood to the UWT and testing even 4 weeks after the trauma in the presence of a reminder cue – is an effective protocol for PTSD-related drug testing, and for neurobiological examination of the neural basis of PTSD.

Importantly, in order to identify potential candidates for pharmacological interventions, we have set out to run a large scale analysis of alterations of target genes expression in the hippocampus and amygdala.

Two complementary approaches were engaged to investigate alterations in expression of target molecules in amygdala and hippocampus after exposure to an odor reminder of underwater trauma (UWT) stress, 4 weeks following the UWT with or without a previous exposure to juvenile stress.

Experiment 1: Long-term effects of UWT on protein expression levels in the BLA and the dorsal versus ventral hippocampus 24h after odor reminder

In this study we began to assess changes in protein expression in the brains of rats that underwent a careful behavioral assessment four weeks after UWT following an odor reminder of the UWT stress, with and without previous juvenile stress. Thereby, we focused on five brain regions, namely the basolateral complex of the amygdala (BLA) as well as the dentate gyrus (DG) and the Cornu Ammonis (CA)1 region of the dorsal and the ventral hippocampus. We plan to analyze the expression of proteins important for inhibitory and excitatory signaling and neuronal plasticity in these regions (Mahan & Ressler, 2012)., starting with the alpha 1 and alpha 2 subunits of the GABA A receptor. We will continue our analysis with GAD65 as a presynaptic marker of GABAergic activity as well as the NMDA receptor subunits NR2A and NR2B to gain insights into excitatory neurotransmission that shapes in balance with inhibitory signaling neuronal plasticity. In this line, we will also assess expression changes in brain-derived neurotrophic factor (BDNF), a key molecule for synaptic plasticity in amygdala and hippocampus that is also associated with posttraumatic stress disorder (Mahan & Ressler, 2012).

Together with the individual behavioral profiles assessed previously this approach will allow for insights into the biochemical basis of certain anxiety- and activity-related behaviors after UWT.

Experiment 1: Methods

Animals

Male Sprague Dawley rats (~22 days old, 30-50 g) were used for the experiments. Animals were housed in groups of ~4, at 22 ± 2°C under 12-h light/dark cycles with water and food available ad libitum. The experiments were approved by the University of Haifa Ethics and Animal Care Committees

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions:

Juvenile and UWT stress exposures + odor reminder [**J+U(+)**] – Rats were exposed to 'juvenile stress' (PND 27-29) and, in adulthood (PND 60), to „UWT stress". 4 weeks following the UWT rats were exposed to the odor 'reminder'.

UWT stress + odor reminder [**UWT(+)**] – Rats were not exposed to 'juvenile stress', but to „UWT stress" in adulthood. 4 weeks following the UWT rats were exposed to the odor 'reminder'.

Control [**Control**] – Rats were neither exposed to 'juvenile stress' nor to „UWT stress" in adulthood, but were exposed to the odor only. 4 weeks following the odor exposure, rats were exposed to the odor 'reminder'.

Experimental design

After five days of acclimatization, rats were randomly assigned to the different experimental groups. Rats in the J+UWT(+) group were exposed to 'Juvenile- stress' at 27-29 PNDs (Horovitz et al., 2012), comprising of 10 min forced swim (PND 27;

in an opaque circular water tank of 0.5m diameter; 0.5m height; 0.4m water depth, water temperature 22±2°C), three trials of elevated platform (PND 28; 30 min. trials; 60 min Inter-Trials Interval (ITI) in the home cage; elevated platform of 12x12cm at 70cm above floor level, located in the middle of a room) and restrainment for 2h under full light illumination (PND 29; metal mesh restraining box of 11x5x4 cm).

In adulthood (~60 PND), J+UWT(+), UWT(+) and Control rats were exposed to the odor delivery cage for 2 min per day on three consecutive days for habituation to the cage. On the 4th day, rats' were exposed to vanilla odor for 30 s and then immediately to the UWT stress, while Control rats were exposed to the odor only. UWT was conducted by placing the rat in a plastic tank. After 5 s of free swimming rats were held under water for 45 s with the help of a special metal net (20x20x15cm; adapted from Wang et al., 2000).

4 weeks following the UWT exposure, J+UWT(+), UWT(+) and Control rats were re exposed to the odor for 30 s in a cage with changed contextual features (colored walls, floor covered with paper tissue instead of bedding) and then were tested in the Open Field for 8 min (OF; Avital and Richter-Levin, 2005; black square plexiglas box of 50×50×38 cm, dim light illumination). 24 hours after the OF test, J+UWT(+), UWT(+) and Control rats were again exposed to the odor and then were tested in the Elevated Plus Maze for 8 min (EPM; Tsoory et al., 2007; two open arms and two closed arms, with 30cm high Plexiglas walls and no roof, 50 cm above the floor). Behavior in OF and EPM tests was recorded and analyzed by EthoVision XT8 tracking system.

24 h after the EPM tests all animals were sacrificed by cervical dislocation, their brains were taken out and immediately snap frozen in powdered dry ice. Brains were stored at -80°C until further processing.

A detailed analyses of behavioral profiles of each animal in EPM and OF was done previously. (See report Ziv. 4.2012)

Harvesting of brain tissue

Brains were mounted on the cerebellum in the cryostat apparatus (chamber temperature -20°C) in a coronar orientation. Slices were chopped until the rostral base of the basolateral amygdala complex (BLA) was reached (-1.8 mm from Bregma; Paxinos & Watson, 1998). With a stainless steal puncher of 1 mm diameter 1.5 mm

long tissue punches were taken from each hemisphere covering the rostro-caudal axis of the BLA. Further slices were taken until the dorsal hippocampus was fully visible (-2.8 mm from Bregma). Here, 2mm long stances of the DG and the CA1 region were harvested bilaterally. The brain was then dismounted form the cryostat holder and re-attached in an horizontal orientation in order to harvest samples from the ventral hippocampus. Starting from the ventral base (-7.6 mm from Bregma), 1.5 mm long punches were taken from the ventral DG and CA1 from each hemisphere, covering their ventro-dorsal axis, hence the venral tip of the hippocampal formation.
All five brain regions were harvested from all rats of the J+UWT(+), UWT(+) and Control group (n=29/ 33/ 34 animals, respectively). All tissue samples were kept frozen during the whole procedure and stored at -80°C until further processing.

Protein sample preparation & Western blot

Frozen tissue samples were homogenized in 300µl Urea Lysis Buffer (1mM EDTA, 0.5% Triton X, 6M Urea, 100µM PMSF) with freshly added protease and phosphotase inhibitor cocktail (complete ultra and PhosStop tablets, Roche Diagnostics, Mannheim , Germany) and incubated at 100°C for 5 min. 10 µg of each sample was loaded for 12 % SDS-polyacrylamide gel for electrophoresis (SDS-PAGE). After semi-dry transfer (nitrocellulose membrane) and blocking of unspecific bindings, incubation with primary antibodies (see Tab. 1) took place over night at 4°C.

Tab. 1-1: Primary antibodies used for western blots

	antibody	**dilution**	**source**
GABA A receptor alpha 1 subunit	rabbit α $GABA_AR$ α1	1:2500	Synaptic systems, Goettingen, Germany
GABA A receptor alpha 2 subunit	rabbit α $GABA_AR$ α1	1:2500	Synaptic systems, Goettingen, Germany
GAD65	mouse α GAD65	1:500	BD Pharmingen, Franklin Lakes, NJ, USA
NMDA receptor 2A subunit	rabbit αNR2A	1:1000	Millipore, Billerica, MA, USA
NMDA receptor 2B subunit	goat NMDAƐ2	1:1000	Santa Cruz, Dallas, TE, USA
BDNF	rabbit αBDNF	1:500	Santa Cruz, Dallas, TE, USA
GAPDH	rabbit α GAPDH	1:2000	Cell Signaling, Beverly, MA, USA

After incubation with complentary secondary antibody (α rabbit, polyclonal 1:15 000; α goat, polyclonal 1:10 000; α mouse, polyclonal 1:10 000) chemiluminescence detection with ECL Plus substrate took place. During densitometric analysis using Quantity One 1-D Analysis software, ratios between the target protein and the housekeeping control protein GAPDH were calculated for each sample and normalized to reference brain sample that was loaded on each gel and allowed for standardization across gels. The optical density was then normalized to the mean density in the Control group for each area.

Statistical Analysis

Differences were determined using one-way or repeated measures analysis of variance (ANOVA). All post hoc comparisons were made using LSD test.

Experiment 1: Results

Long-term effects of UWT on protein expression levels in the BLA and the dorsal versus ventral hippocampus 24h after odor reminder

UWT in combination with juvenile stress affected the protein expression levels of GABA A receptor alpha 1 and alpha 2 in a region-specific manner (Fig. 1-2). One-way ANOVA for treatment group for each region followed by LSD post hoc comparison indicated a significant increase of the alpha 1 subunit expression in the ventral DG ($F(2,93)=3.368$; $p=0.039$; $p<0.05$ compared to Control), but not in the dorsal DG ($F(2,93)=2.130$; $p=0.125$). In the BLA, alpha 1 subunit expression was elevated in the J+U(+) group as well ($F(2,93)=5.267$; $p=0.007$; $p<0.01$ compared to Control; $p<0.05$ compared to U(+)). Similar but insignificant trends were observed for the dorsal and ventral CA1 region (dCA1: $F(2,69)=2.603$; $p=0.81$; vCA1: $F(2,93)=2.978$; $p=0.056$).

Expression levels of the alpha 2 subunit were also increased in the ventral DG ($F(2,93)=9.478$; $p<0.0001$), but in none of the other regions examined (dDG: $F(2,45)=0.229$; $p=0.796$; dCA1: $F(2,69)=1.021$; $p=0.366$; vCA1: $F(2,93)=1.424$; $p=0.246$; BLA: $F(2,45)=1.295$; $p=0.284$). As shown by LSD post hoc comparison,

alpha 2 subunit protein expression was enhanced after UWT alone ($p<0.05$) and after its combination with juvenile stress ($p<0.001$) in the vDG.

Thus, GABA A receptor subunits displays a region-specific expression pattern after severe stress, with a great diversity especially in the ventral versus dorsal DG.

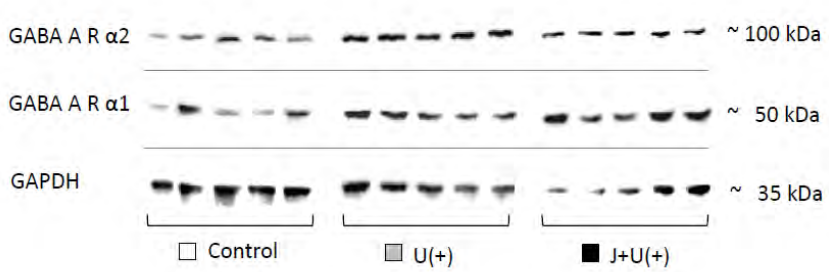

Fig. 1-1: Example immunoblots for GABA receptor alpha 1 and 2 subunit protein expression in the ventral dentate gyrus four weeks after UWT with odor reminder (U(+)) and with additional juvenile stress (J+U(+)). The housekeeping protein GAPDH was used as internal control.

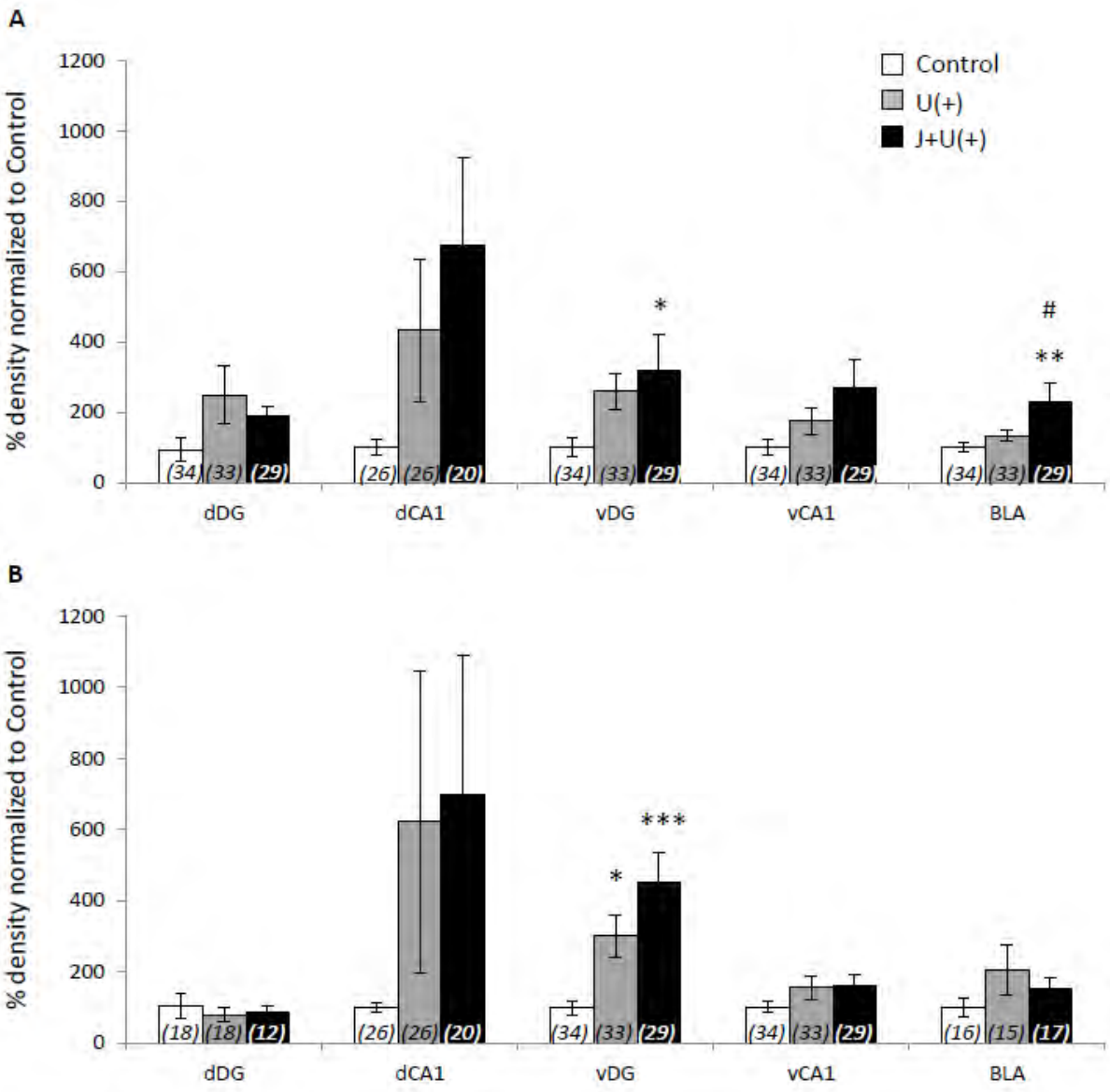

Fig. 1-2: Expression of neuropeptides 4 weeks after UWT with odor reminder (U(+)) and with additional juvenile stress (J+U(+)). (A) Juvenile stress in combination with underwater trauma (UWT) induced a long-term upregulation of the GABA receptor alpha 1 subunit protein expression in the ventral but not dorsal dentate gurys (DG) and in the basolateral complex of the amygdala (BLA). In trend, this pattern was also visible for the ventral and dorsal CA1. (B) UWT, with and without juvenile stress, increased the expression of the the GABA receptor alpha 2 subunit in the ventral DG only. All values optical density normalized to mean of control group, mean ±SEM. (n) Number of animals analyzed. * significant difference to control group, $p<0.05$; ** $p<0.01$; $p<0.001$; # significant difference to U(+), $p<0.05$.

Experiment 2: Long-term effects of UWT on mRNA expression levels in sublayers of the dorsal dentate gyrus (DG) and the BLA

Aim of this study is the investigation of the impact of the UWT, with and without additional juvenile stress, on expression of target genes related to inhibitory signaling. Using laser capture microdissection and quantitative real time PCR, gene expression profile of subareas of brain regions will add further insights in stress-related molecular alteration on a high resolution level. Of special interest is the dorsal DG as the input station for incoming information into the hippocampal formation. As a highly organized structure, the DG receives inputs from the entorhinal cortex into the outer two-thirds of its molecular layer. The granule cell layer contains the cell bodies of the primary excitatory neurons in the DG, sending their axons trough the hilus region towards the CA3 region of the hippocampus (Ascadi & Kali, 2007). All three subregions are rich on a diversity of GABAergic interneurons (Houser, 2007), with in part specialiced locations and contents of neuropeptides, acting as GABAergic co-transmitters and neuromodulators (Freund & Buzsáki, 1996). These interneurons excert strong feedforward and feedback inhibition, shaping thereby the information flow in the hippocampal formation (Ascadi & Kali, 2007). We now started to analyze the expression of the neuropeptides cholecystokinin (CCK), somatostatin (SST) and neuropeptide Y (NPY) as well as the inhibitory GABA A receptor subunits alpha 1 and 2, GAD65 and GAD67 and the excitatory NMDA receptor subunits NR2A and NR2B in sublayers of the dorsal DG as well as in the BLA, a region that is known to modulate DG plasticity (Vouimba & Richter-Levin, 2005), after UWT.

Experiment 2: Methods

Animals

Male Sprague Dawley rats (~22 days old, 30-50 g) were used for the experiments. Animals were housed in groups of ~4, at 22 ± 2°C under 12-h light/dark cycles with water and food ad libitum. The experiments were approved by the University of Haifa Ethics and Animal Care Committees

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions:

Juvenile and UWT stress exposures + odor reminder [**J+U(+)**] – Rats were exposed to 'juvenile stress' (PND 27-29) and, in adulthood (PND 60), to „UWT stress". 4 weeks following the UWT rats were exposed to the odor 'reminder'.

UWT stress + odor reminder [**UWT(+)**] – Rats were not exposed to 'juvenile stress', but to „UWT stress" in adulthood. 4 weeks following the UWT rats were exposed to the odor 'reminder'.

Control [**Control**] – Rats were neither exposed to 'juvenile stress' nor to „UWT stress" in adulthood, but were exposed to the odor only. 4 weeks following the odor exposure, rats were exposed to the odor 'reminder'.

Experimental design

Essentially, experiments were conducted as described in experiment 1. However, four weeks after UWT, rats were re-exposed to the odor as described before and immediately tested in the EPM. In contrasts to experiment 1, animals were then sacrificed immediately after the behavioral test in order to avoid effects of EPM exposure on gene expression.

The brains were taken out and snap frozen immediately in powdered dry ice and stored at -80°C until further processing.

Laser capture microdissection (LCM) and RNA isolation

20µm thick coronar sections were cut on a cryostat at the level of the dorsal hippocampus and BLA (-2.3 mm – -4.16 mm from Bregma; Paxinos & Watson, 1998) and thaw mounted on poly-L-lysine coated RNase-free membrane slides (Leica Microsystems, Wetzlar, Germany). After fixation in -20°C cold 70 % ethanol and brief cresyl violet acetate staining under nuclease-minimized conditions, the outer 2/3^{rds} of the molecular layer, the granule cell layer and the hilus of the dorsal DG as well as the BLA were microdissected from 10-14 sections per animal and collected in the cap of a RNAse-free plastic tube using a laser capture microdissection system (Leica Microsystems, Wetzlar, Germany). Sample lysis and subsequent isolation of

total RNA via a spin column system was conducted with the RNeasy Micro Plus kit (Qiagen, Hilden, Germany) according to manufacturer‟s instructions, including steps for removal of genomic DNA.

Reverse transcription and real time PCR

First-strand synthesis of cDNA was performed with the Sensiscript Reverse Transcription kit (Qiagen, Hilden, Germany), specifically designed for low amounts of RNA, in the presence of 2.5mM dNTPs, 50µM Oligo (dT)18 and 50 µM random decamer first strand primers (Life Technologies, Carlsbad, CA, USA) as well as RNase Inhibitor (SuperaseIN; 20 U/µl; Life Technologies, Carlsbad, CA, USA) for 60 min at 37°C. A 1:5 dilution of cDNA samples was used for determination of target gene expression levels via quantitative PCR using the ABI Prism Step One real time PCR apparatus (Life Technologies, Carlsbad, CA, USA) and TaqMan® reagents with predesigned assays for target genes (see Tab. 2-1) and the housekeeping gene glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH; endogenous control, assay ID: Rn_99999916_s1, Life Technologies, Carlsbad, CA, USA). Target and housekeeping genes were labeled with different fluorescent dyes, allowing for quantitative multiplex PCR.

Tab. 2-1 TaqMan gene expression assays used (all predesigned by Life Technologies, Carlsbad, CA, USA).

Target gene	**assay ID**
Cholecystokinin (CCK)	Rn00563215_m1
Neuropeptide Y (NPY)	Rn01410145_m1
somatostatin (SST)	Rn00561967_m1
GAD65	Rn00561244_m1
GAD67	Rn00690300_m1
GABA A receptor alpha 1 subunit (Gabra1)	Rn00788315_m1
GABA A receptor alpha 2 subunit (Gabra2)	Rn01413643_m1
NMDA receptor 2A subunit (Grin2a)	Rn00561341_m1
NMDA receptor 2A subunit (Grin2b)	Rn00680474_m1
BDNF	Rn02531967_s1

All samples were run in triplicate assays, consisting of 50 cycles of 15s at 95°C and 1min at 60°C, preceded by a 2min decontamination step at 50°C with Uracil-N-Glycosidase and initial denaturation at 95°C for 10 min.

For data analysis, the mean cycle treshold (CT) was determined for each triplicate assay and relative quantification of each target gene was conducted with the ddCT method (Livak and Schmittgen, 2001), normalizing each sample to the overall content of cDNA using GAPDH as an internal control (dCT; dCT = dCT (target gene) = (CT (target gene)) - (CT (GAPDH)). Normalization of all ddCT values was done relative to control group with ddCT= dCT(sample) – mean dCT (control group). Transformation to RQ values for a specific target gene and area was done according to $RQ=2^{-ddCT}$ with RQ%(control)=100.

Statistical analysis.

ANOVA for group followed by LSD tests for *post-hoc* comparison was done for each region and target gene.

<u>Experiment 2:</u> Results – preliminary:

Long-term effects of UWT & Juvenile stress on neuropetide mRNA expression levels in sublayers of the dentate gyrus and the BLA

All animals in this experiment were exposed to an odor reminder cue of the UWT trauma and tested in the EPM four weeks after the UWT and were sacrificed immediately after. This allowed for assessing the behavioral impact of the UWT, with or without previous juvenile stress experience, on activity and anxiety-like behavior (Fig. 2-1).

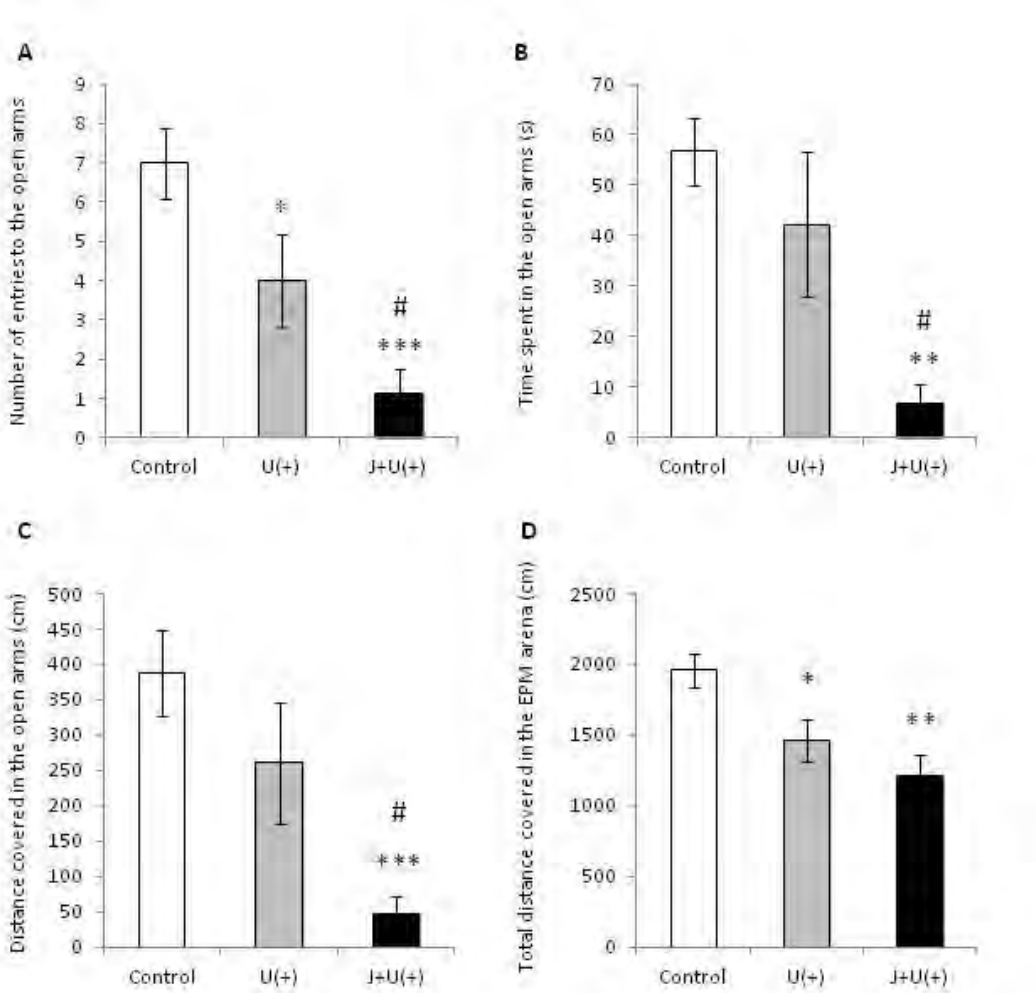

Fig. 2-1: In the rats assigned for analysis of gene expression in sublayers of the dentate gyrus and the basolateral amygdala, previous effects on anxiety like behavior and activity in the elevated plus maze were confirmed. (A) Four weeks after UWT, immediately after re-exposure to an odor reminder cue (U(+)), entries to the open arms were diminished. Such anxiety-like behavior was further increased when rats experienced juvenile stress (J+U(+)). (B) The UWT with previous juvenile stress experience reduced the time spent in the open arms as well as (C) distance covered in the open arms. (D) The general distance covered in the maze was also reduced after UWT and its combination with juvenile stress. All values mean ±SEM. * significant difference to Control, $p<0.05$; ** $p<0.01$; *** $p<0.001$. # significant difference to U(+), $p<0.05$.

One-way ANOVA demonstrated significant differences between the groups for entries to the open arm ($F(2,23)=10.755$; $p=0.001$), time spent in the open arm ($F(2,23)=8.511$; $p=0.002$) and distance covered in the open arms ($F(2,23)=8.472$; $p=0.002$). LSD post hoc analysis revealed a significant reduction in open arm entries when UWT was applied four weeks before ($p<0.05$), that was further decreased when rats experienced juvenile stress ($p<0,001$ compared to Control; $p<0.05$ compared to U(+)). However, only the combination of juvenile and UWT stress reduced the time spent ($p<0,01$ compared to Control; $p<0.05$ compared to U(+)) and the distance covered ($p<0,001$ compared to Control; $p<0.05$ compared to U(+)) in open arms significantly. The general activity as indicated by total distance covered in the maze was reduced after UWT ($p<0.05$) and further after juvenile stress and UWT ($p<0.01$). Preliminary analysis of gene expression, focusing on neuropeptides, revealed a subregion specific impact of UWT and juvenile stress (Fig. 2-2).

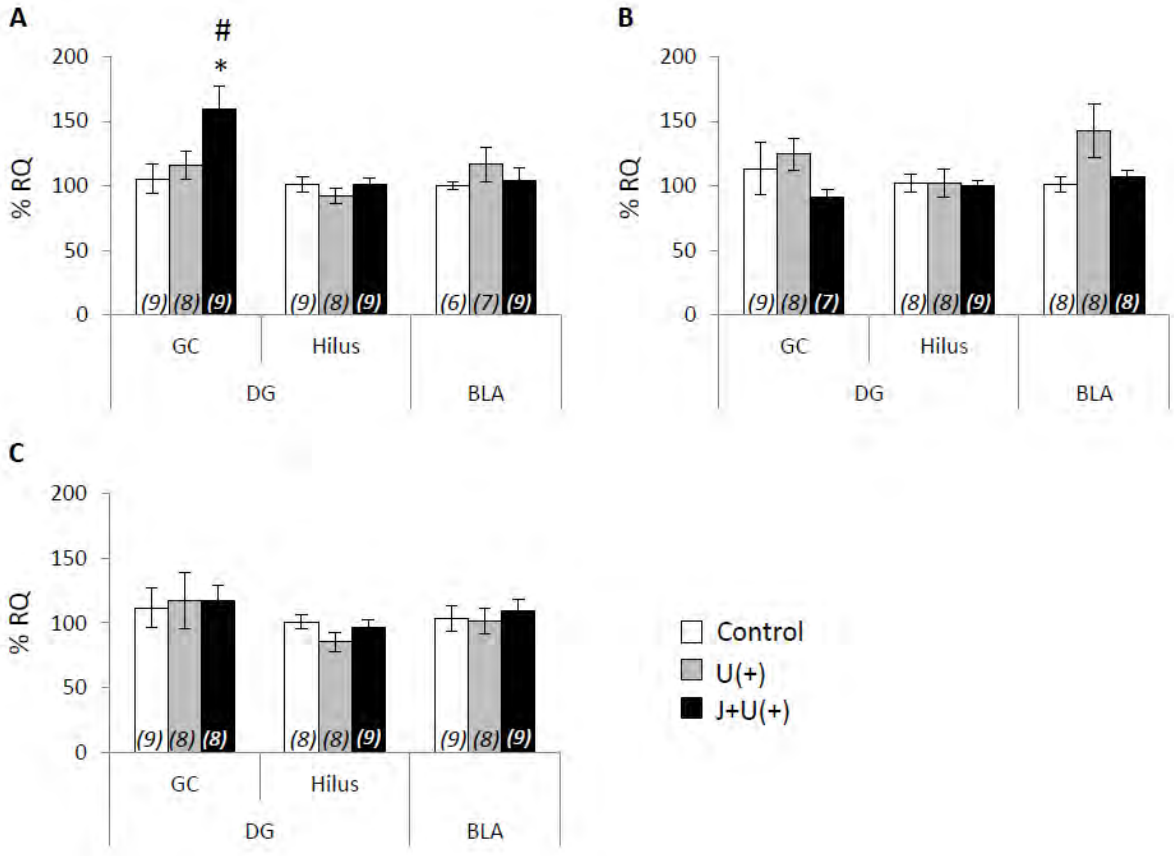

Fig. 2-2: Expression of neuropeptides 4 weeks after UWT with odor reminder (U(+)) and with additional juvenile stress (J+U(+)). (A) Juvenile stress in combination with underwater trauma (UWT) induced a long-term upregulation of of cholecystokinine (CCK) mRNA levels in the granule cell (GC) layer of the dentate gyrus (DG), but not in the hilus or in the basolateral complex of the amygdala (BLA). (B) In trend, neuropeptide Y (NPY) expression was increased in the BLA after UWT, but not in combination with juvenile stress. (C) No expression changes were observed for somatostatin (SST) in the DG or the BLA. All values relative expression (%RQ) to mean of control group, mean ±SEM. (n) Number of animals analyzed per group. * significant difference to control group, $p<0.05$; # significant difference to U(+), $p<0.05$.

One-way ANOVA followed by LSD post hoc comparison examining group effects for each subregion and target gene demonstrated an increase in CCK mRNA expression levels after UWT in combination with juvenile stress only in the granule cell layer of the DG ($F(2,23)=4.208$; $p=0.028$; $p<0.05$ compared to Control and to U(+)), but not in the hilus ($F(2,23)=0.915$; $p=0.415$) or in the BLA ($F(2,19)=0.529$; $p=0.658$).

For NPY, ANOVA showed a strong trend of altered expression in the BLA ($F(2,21)=3.175$; $p=0.062$), with increased NPY mRNA levels after UWT, but not when combined with juvenile stress. NPY expression in the DG sublayers was not altered (GC: $F(2,21)=1.102$; $p=0.351$; Hilus: $F(2,22)=0.013$; $p=0.987$).

Long-term expression of SST was not affected by UWT or its combination with juvenile stress in any of the regions investigated (GC: $F(2,22)=0.037$; $p=0.964$; Hilus: $F(2,22)=1.397$; $p=0.268$; BLA: $F(2,23)=0.182$; $p=0.835$).

Task 6: **Assessing juvenile stress pre-disposing effects on sensitivity to sleep restriction in adulthood –**

Because of the issues that came up with regards to the SR protocol, as described in tasks 2,4 above, we will continue to investigate the SR protocol, as described above prior to progressing with Task 6. Instead, as is described in Task 3,5, we made very good progress with the 'juvenile' and adult trauma model, and so will focus in the meantime on this protocol also with regards to task 7.

Key research accomplishments

Towards the end of the second year of the project, the following can already be indicated as research accomplishments:

- The UWT model, which is an ethological model of a brief but intense traumatic event (Richter-Levin, 1998) was further developed here in a way that is of particular relevance to combat soldiers. It was found to have an impact by itself, but to be a convenient platform for examining the added impact of relevant risk factors.
- The maladaptive response of PTSD patients to reminder cues of the traumatic events is a hallmark of the disorder. We have incorporated this important component into our model. This will enable us
  a) To utilize the sensitivity of the model as a drug testing platform.
  b) To better understand variables which contribute to the effectiveness of reminder cues (in order to guide treatment).
  c) To use the model to elucidate the neural mechanisms associated with abnormal responses to reminder cues.
- A rat model of high relevance to PTSD was confirmed (task 5). That finding that PTSD symptoms in this model last for over four weeks establishes it as a relevant model but also enables utilizing this model for long-term drug treatment at different time points following the exposure to the traumatic event.

Reportable outcomes

Published Manuscripts (in which the support of the DOD is indicated):

Sood R, Ritov G, **Richter-Levin G**, Barki-Harrington L. (2012) Selective increase in the association of the β2 adrenergic receptor, β Arrestin-1 and p53 with Mdm2 in the ventral hippocampus one month after underwater trauma. Behavioral Brain Research. 240:26-8.

Horovitz O, Tsoory MM, Yovell Y, **Richter-Levin G.** (2012) A rat model of pre-puberty (Juvenile) stress-induced predisposition to stress-related disorders: Sex similarities and sex differences in effects and symptoms. World Journal of Biological Psychiatry (in press).

Manuscripts in preparation:

1) Ardi Z., Ritov, G., Lucas M. and Richter-Levin G., The effects of a reminder of underwater trauma on behavior and memory-related mechanisms in the rat dentate gyrus.
2) Ritov, G. Ardi, Z., and Richter-Levin, G., Differential activation of dorsal and ventral hippocampus and amygdala following an exposure to a reminder of underwater trauma.

Abstracts in meetings:

Ardi Z., Richter-Levin A. and Richter-Levin G. (2012). 'Juvenile stress' exacerbates the impact of an exposure to a reminder of a stressful experience in adulthood, The 16th Annual meeting of the Israeli society for biological psychiatry, Israel.

Hadad O, and Richter-Levin G. (2012) Reflection of behavioral on gene expression in Dorsal and Ventral Hippocampus and the Amygdala in response to stress in Juvenility and Adulthood. The 16th annual meeting of the Israel society for biological psychiatry. Kibbutz Hagoshrim, Israel.

Ariel L., Faraggi M., and Richter-Levin G. (2012) Acute stress alters daily rhythm of body temperature while sleep restriction does not exacerbate this influence. The 21st international congress of Zoology. Haifa. Israel.

Ariel L., Faraggi M., and Richter-Levin G. (2012) The effects of stress in adulthood, following sleep restriction or stress in juvenility, on body temperature and activity level. The 21st Annual meeting of the Israel society for neuroscience, Israel.

Ashkenazi-Karni, S., Horovitz, O, Metzger, N, Vered, R, Anunu, R. and Richter-Levin, G (2012) Exposure to stress differentially affects behavior and expression of kappa opioid receptor and GABA-A receptor in male rats. The 21st Annual meeting of the Israel society for neuroscience, Israel.

Horovitz, O., Ashkenazi-Karni, S. and Richter-Levin, G. (2012) Stress exposure and GABA-Aα2 receptor protein expression in emotional neural circuits. The 21st Annual meeting of the Israel society for neuroscience, Israel.

MinXin Fan, Li Xia, Martin Kriebel, Hansjürgen Volkmer, Gal Richter-Levin (2012) GABAa receptor α2 but not α1 subunit knockdown in dentate gyrus reduces inhibition activity and improves learning abilities of rats. The 21st Annual meeting of the Israel society for neuroscience, Israel.

Ardi Z., Richter-Levin A. and Richter-Levin G. (2012). 'Juvenile stress' exacerbates the impact of an exposure to an odor reminder of a stressful experience in adulthood. Classification of the behavioral effects, The 21st Annual meeting of the Israel society for neuroscience, Israel.

Sood, R1,. Ritov, G1., Richter-Levin, G and Barki-Harrington, L.(2012). Selective increase in the association of the Beta 2 adrenergic receptor, Beta Arrestin-1 and p53 with Mdm2 in the ventral hippocampus one month after underwater trauma. The 21th Annual Meeting of Israel Society for Neuroscience, Israel.

Horovitz,O., Strominger, I., Ashkenazi-Karni, S., and Richter-Levin, G. (2012) Exposure to stress differentially affects behavior and brain activity in male and female rats. Frontiers in Stress and Cognition: From Molecules to Behavior conference, Ascona, Switzerland.

Ardi Z., Richter-Levin A. and Richter-Levin G. (2012). Classification of the behavioral effects of an exposure to a reminder of a stressful experience in adulthood following an exposure to 'juvenile stress', Frontiers in Stress and Cognition: From Molecules to Behavior conference, Switzerland.

Shtoots L., Anunu R. and Richter-Levin G. (2012) "Juvenile stress: impairment and resilience are reflected in peritoneal inflammatory response". The 8th FENS forum of neuroscience. Barcelona, Spain.

Hadad O., Albrecht A., Stork O., and Richter-Levin G. (2012) Differential Effect of 'Controllable vs. Uncontrollable' Stress on Amygdala Activation and Gene Expression in Hippocampal Sub Regions. The 8th FENS forum of neuroscience. Barcelona, Spain.

Ritov, G., Ardi, Z., and Richter-Levin, G.(2012). Differential activation of dorsal and ventral hippocampus and amygdala following an exposure to a reminder of underwater trauma. The 8th FENS forum of neuroscince, Barcelona, Spain.

Shtoots L., Hugeri O., Anunu R. and Richter-Levin G. (2013) "Enriched environment effects on juvenile stress-induced alterations of CCL2 and CCR2 expression following induced peritonitis in rats". The 17th annual meeting of the Israel society for biological psychiatry. Kibbutz Hagoshrim, Israel.

Horovitz, O. and Richter-Levin, G. (2013) Dorsal Periaqueductal gray (dPAG) modulation of plasticity in the Nucleus Accumbens (NAcc) and the Basloateral Amygdala (BLA) induced by Ventral Subiculum (vSub) stimulation. The 17th annual meeting of the Israel society for biological psychiatry. Kibbutz Hagoshrim, Israel.

Ardi Z., Richter-Levin A. and Richter-Levin G. (2013). Preventing predisposition to stress induced anxious behaviors by 'Enriched Environment' in juvenility – a platform to study resilience. The 17th Annual meeting of the Israeli society for biological psychiatry, Israel.

Anne Albrecht, Sebastian Ivens, Uwe Heinemann, Gal Richter-Levin and Oliver Stork (2013) Allosteric regulation of neuron-astrocyte interaction in the dentate gyrus in an animal model of post traumatic stress disorder. The 17th Annual meeting of the Israeli society for biological psychiatry, Israel.

Ritov, G., Boltyansky, B., and Richter-Levin, G.(2012). The effects of exposure to a contextual reminder four weeks after underwater trauma. The 17th Israel Society For Biological Psychiatry Meeting, Israel.

Conclusions

This report is of the second year of a 4 years project. The project is set to examine the impact of two risk factors (Childhood stress and sleep restriction) for the development of PTSD, and to establish an effective platform for drug testing.

We have made excellent progress in establishing childhood stress as a risk factor, and in identifying neural mechanisms associated with this risk factor, that may serve as novel potential targets for developing new drugs. This goal will continue into the 3rd and 4th years.

We have begun to explore the contribution of sleep restriction on development of PTSD. Here progress is slow, mainly since the protocol that was adopted from Meerlo et al, 2008 was not found to be effective. As is indicated in the report, we have conducted methodological changes and have started to assess their effectiveness. This goal will continue into the 3rd and 4th years.

We have established an effective platform for drug testing. We have started to get organized for conducting drug testing, to further validate the procedure and this goal will continue into the 3rd and 4th years.

First papers were published but a number of manuscripts are currently in preparation.

It can be stated that progress is good and that the main aims of the project will be achieved as planned.

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